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(54) **MUTANT T7 POLYMERASES**

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C12N 9/12 (2006.01)

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CPC *C12N 9/1247* (2013.01)

(58) **Field of Classification Search**
None
See application file for complete search history.

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(57) **ABSTRACT**

Provided are mutant polymerases that comprise a deletion of at least four amino acids among the amino acids at positions corresponding to 167-174 of SEQ ID NO:1. Also provided are mutant polymerases having greater resistance to 30 mM NaCl, 7.5 mM phosphate, or 20 μg/ml single stranded DNA than a wild-type T7 RNA polymerase having SEQ ID NO:1 or a wild-type T3 RNA polymerase having SEQ ID NO:3. Nucleic acids comprising a nucleotide sequence encoding any of the above mutant polymerases are also provided, as are vectors comprising those nucleic acids and host cells transformed with the vectors Additionally, methods of amplifying mRNA using the mutant polymerases described herein are also provided. Further, compositions comprising any of the mutant polymerases described herein, and a reagent at a concentration that is inhibitory to wild-type T7 RNA polymerase is provided.

12 Claims, 3 Drawing Sheets

Identities = 726/884 (82%), Positives = 795/884 (89%), Gaps = 1/884 (0%)

T3	1	MNIIENIEKNDFSEIELAAIPFNTLADHYGSALAKEQLALEHESYELGERRFLKMLERQA	60
T7	1	MNTI-NIAKNDFSDIELAAIPFNTLADHYGERLAREQLALEHESYEMGEARFRKMFERQL	59
T3	61	KAGEIADNAAAKPLLATLLPKLTTRIVEWLEEYASKKGRKPSAYAPLQLLKPEASAFITL	120
T7	60	KAGEVADNAAAKPLITTLLPKMIARINDWFEEVKAKRGKRPTAFQFLQEIKPEAVAYITI	119
T3	121	KVILASLTSTNMTTIQAAAGMLGKAIEDEARFGRIRDLEAKHFKKHV**EEQLNKRH**GQVYK	180
T7	120	KTTLACLTSADNTTVQAVASAIGRAIEDEARFGRIRDLEAKHFKKNV**EEQLNKRV**GHVYK	179
T3	181	KAFMQVVEADMIGRGLLGGEAWSSWDKETTMHVGIRLIEMLIESTGLVELQRHNAGNAGS	240
T7	180	KAFMQVVEADMLSKGLLGGEAWSSWHKEDSIHVGVRCIEMLIESTGMVSLHRQNAGVVGQ	239
T3	241	DHEALQLAQEYVDVLAKRAGALAGISPMFQPCVVPPKPWVAITGGGYWANGRRPLALVRT	300
T7	240	DSETIELAPEYAEAIATRAGALAGISPMFQPCVVPPKPWTGITGGGYWANGRRPLALVRT	299
T3	301	HSKKGLMRYEDVYMPEVYKAVNLAQNTAWKINKKVLAVVNEIVNWKNCPVADIPSLERQE	360
T7	300	HSKKALMRYEDVYMPEVYKAINIAQNTAWKINKKVLAVANVITKWKHCPVEDIPAIEREE	359
T3	361	LPPKPDDIDTNEAALKEWKKAAAGIYRLDKARVSRRISLEFMLEQANKFASKKAIWFPYN	420
T7	360	LPMKPEDIDMNPEALTAWKRAAAAVYRKDKARKSRRISLEFMLEQANKFANHKAIWFPYN	419
T3	421	MDWRGRVYAVPMFNPQGNDMTKGLLTLAKGKPIGEEGFYWLKIHGANCAGVDKVPFPERI	480
T7	420	MDWRGRVYAVSMFNPQGNDMTKGLLTLAKGKPIGKEGYYWLKIHGANCAGVDKVPFPERI	479
T3	481	AFIEKHVDDILACAKDPINNTWWAEQDSPFCFLAFCFEYAGVTHHGLSYNCSLPLAFDGS	540
T7	480	KFIEENHENIMACAKSPLENTWWAEQDSPFCFLAFCFEYAGVQHHGLSYNCSLPLAFDGS	539
T3	541	CSGIQHFSAMLRDEVGGRAVNLLPSETVQDIYGIVAQKVNEILKQDAINGTPNEMITVTD	600
T7	540	CSGIQHFSAMLRDEVGGRAVNLLPSETVQDIYGIVAKKVNEILQADAINGTDNEVVTVTD	599
T3	601	KDTGEISEKLKLGTSTLAQQWLAYGVTRSVTKRSVMTLAYGSKEFGFRQQVLDDTIQPAI	660
T7	600	ENTGEISEKVKLGTKALAGQWLAYGVTRSVTKRSVMTLAYGSKEFGFRQQVLEDTIQPAI	659
T3	661	DSGKGLMFTQPNQAAGYMAKLIWDAVSVTVVAAVEAMNWLKSAAKLLAAEVKDKKTKEIL	720
T7	660	DSGKGLMFTQPNQAAGYMAKLIWESVSVTVVAAVEAMNWLKSAAKLLAAEVKDKKTGEIL	719
T3	721	RHRCAVHWTTPDGFPVWQEYRKPLQKRLDMIFLGQFRLQPTINTLKDSGIDAHKQESGIA	780
T7	720	RKRCAVHWVTPDGFPVWQEYKKPIQTRLNLMFLGQFRLQPTINTNKDSEIDAHKQESGIA	779
T3	781	PNFVHSQDGSHLRMTVVYAHEKYGIESFALIHDSFGTIPADAGKLFKAVRETMVITYENN	840
T7	780	PNFVHSQDGSHLRKTVVWAHEKYGIESFALIHDSFGTIPADAANLFKAVRETMVDTYESC	839
T3	841	DVLADFYSQFADQLHETQLDKMPPLPKKGNLNLQDILKSDFAFA	884
T7	840	DVLADFYDQFADQLHESQLDKMPALPAKGNLNLRDILESDFAFA	883

FIG. 1

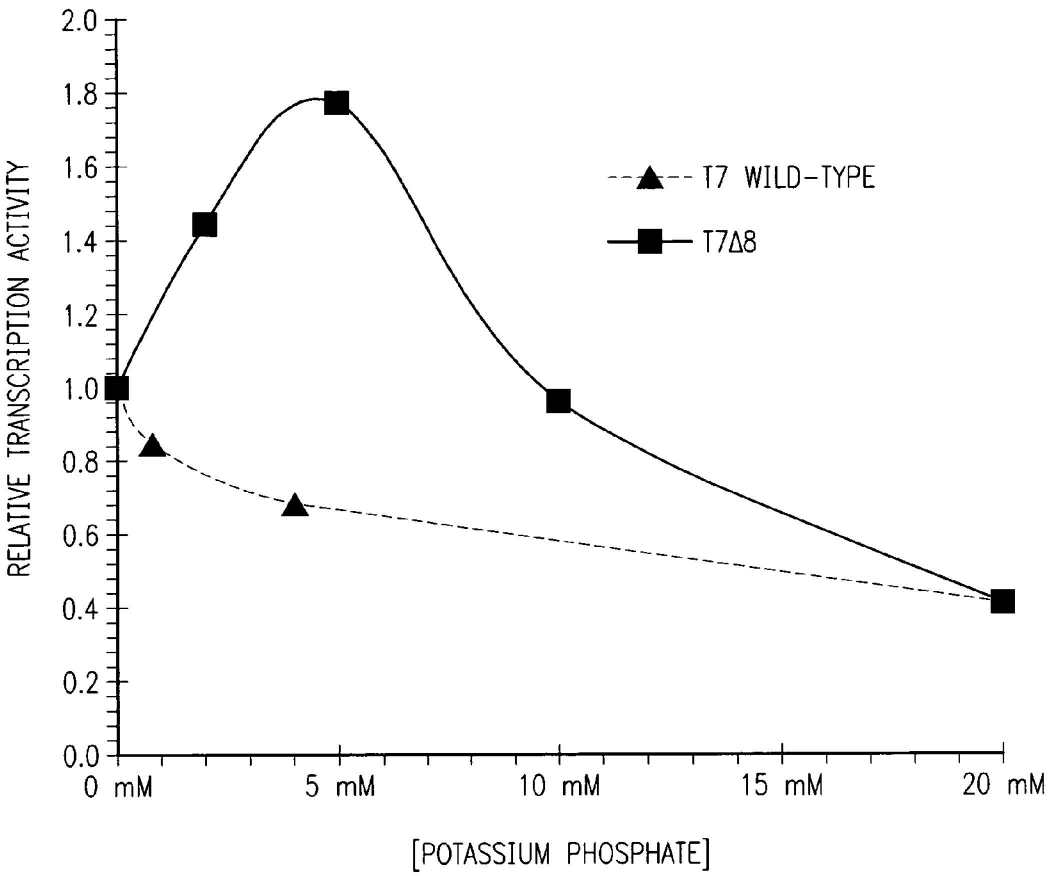
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
RELATIVE TRANSCRIPTION ACTIVITY
T7 WILD-TYPE
T7Δ8
0 mM
5 mM
10 mM
15 mM
20 mM
[POTASSIUM PHOSPHATE]

FIG. 2

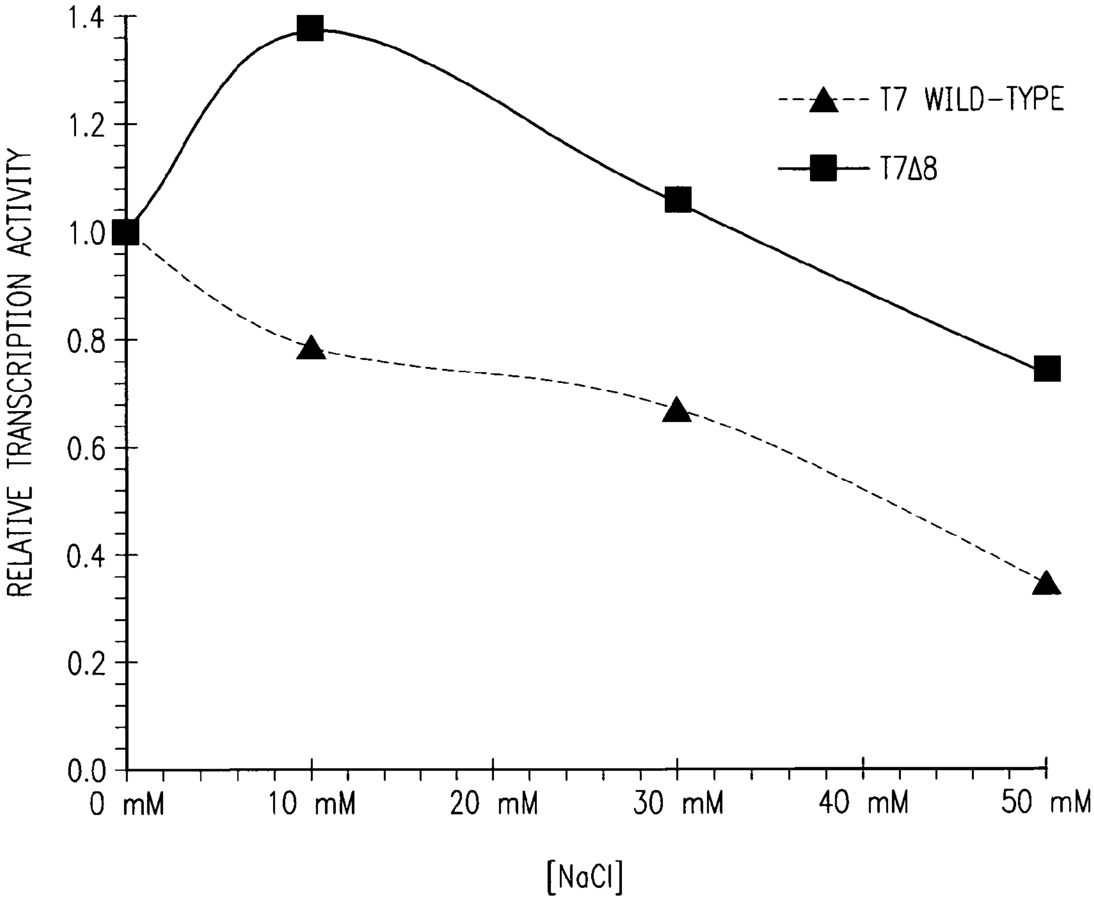
RELATIVE TRANSCRIPTION ACTIVITY
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
T7 WILD-TYPE
T7Δ8
0 mM
10 mM
20 mM
30 mM
40 mM
50 mM
[NaCl]

FIG. 3

MUTANT T7 POLYMERASES

BACKGROUND OF THE INVENTION

(1) Field of the Invention

The present invention generally relates to improved enzymes for molecular biology. More specifically, mutant RNA polymerases are provided that have improved resistance to common reagents including phosphate.

(2) Description of the Related Art

RNA and DNA polymerization reactions, which result in the synthesis of RNA or DNA polynucleotides, are an integral part of a variety of techniques used in molecular biology. Such reactions include in vitro transcription and amplification techniques such as the polymerase chain reaction (PCR), RNA amplification and self-sustained sequence replication. These reactions often employ RNA polymerases, especially bacteriophage RNA polymerases such as SP6, T7 and T3, for example, in the synthesis of both labeled RNA probes and unlabeled RNA. Improved performance of the RNA polymerases utilized in these reactions would thus be beneficial.

The rate of these synthetic reactions, and the amount of product formed, is limited by several factors. Lowering the magnesium concentration and salt concentration allows the use of high concentrations of substrate nucleotides and improve the yields of a transcription reaction (U.S. Pat. Nos. 6,586,219, 6,586,218 and 5,256,555). Those techniques prevent inhibition caused by some reaction substrates, but inhibition by other substrates and reaction products, e.g., phosphate, pyrophosphate and single stranded DNA (ssDNA) can still inhibit polymerase activity.

Transcription reactions and DNA polymerase and DNA sequencing applications routinely use the enzyme inorganic pyrophosphatase since addition of that enzyme improves the yield of transcription reactions by removing pyrophosphate (Sampson & Uhlenbeck, 1988; Weitzmann et al., 1990; Cunningham & Ofengand, 1990; Tabor & Richardson, 1990). Pyrophosphatase cleaves the polymerase reaction product pyrophosphate to produce two molecules of phosphate. However, phosphate inhibits RNA polymerase, especially at high concentrations. For example, the optimal total concentration of nucleotides found by Cunningham & Ofengand (1990) of 16 mM produces 32 mM phosphate at the end of the reaction, which is inhibitory to RNA polymerase.

Although there are various protocols in molecular biology where reactions utilizing more than one enzyme are combined, the inhibition of RNA polymerases by reagents such as pyrophosphate and phosphate can thwart efforts to simplify protocols. For example, a typical protocol for amplification of mRNA involves synthesizing a first strand cDNA using reverse transcriptase, followed by a second strand cDNA synthesis using DNA polymerase, then RNA transcription from the cDNA using RNA polymerase. See, e.g., Wang et al., 2000. These protocols usually require a cDNA purification step after the second strand synthesis because buffers and reaction products present from the cDNA synthesis procedures inhibit the RNA polymerase. Some second strand synthesis buffers are available that do not have phosphate, but an RNA polymerase that is not inhibited by phosphate would make their use, or a cDNA purification step, unnecessary.

One of the characteristics of wild-type T7 RNA polymerase is the ability to carry out some level of promoter independent synthesis by using the 3' ends of single-stranded DNA as an initiation site. For in vitro transcription reactions, substrates that give this synthesis can be DNA primers and double-stranded linearized DNA with single-stranded 3' tails. This can especially be a problem when there are large amounts of primers present and low levels of promoter templates. This can also take place with low amounts of RNA analyte samples where single-stranded carrier DNA has been added to increase efficiency of recovery. As a consequence of this property, there can be a large amount of aberrant synthesis taking place even in the complete absence of any input RNA, thus implying that at low levels of legitimate targets, a large amount of labeled product is only contributing to background and not signal. Secondly, the formation of this promoter independent synthesis uses up reagents such that the net yield of legitimate product can be decreased by competition with the promoter independent synthesis.

A number of mutations in RNA polymerases that modify characteristics of those enzymes are known. For example, certain mutations in T7 RNA polymerase ("T7") (e.g., Y639F/S641A; del172-173; F644Y; F667Y) allow the polymerase to utilize deoxyribonucleotides along with ribonucleotides as substrates (Kostyuk et al., 1995; Izawa et al., 1998; European Patent Application EP1403364A1). See also Joyce (1997), Izawa et al. (1998) and Brieba and Sousa (1999). Other mutations increase (e.g. K172L, Del172-173, K98R) or decrease (e.g., P266L) promoter binding strength (Tunitskaya and Lochetkov, 2002; U.S. Pat. No. 7,335,471) or alter the termination properties of the enzyme (e.g., del 163-164, R173C). See Lyakhov et al. (1992), Lyakhov et al. (1997), Tunitskaya and Lochetkov (2002). Still other mutations (e.g., N748D, N748Q, Q758C, E222K, R756M) alter promoter recognition (U.S. Pat. No. 5,385,834; Chilliserry-lattil et al., 2001) or increase the thermostability of the enzyme (e.g., S430P, F849I, F880Y, S633P—U.S. Pat. No. 7,507,567). Additional T7 mutations are described in He (1996), Macdonald et al. (1994), and Yang and Richardson (1997).

Mutations analogous to some of the above mutations have been effectively made in T3 RNA polymerase ("T3") (see, e.g., Lyakhov et al., 1997 and European Patent Application No. EP1403364), demonstrating that the various domains of these related phage RNA polymerases are functionally equivalent.

The present invention provides, in part, RNA polymerase mutants with improved characteristics, including resistance to phosphate, pyrophosphate, sodium chloride, and/or single stranded DNA that can be advantageously used in place of wild-type RNA polymerases for various molecular biology procedures.

BRIEF SUMMARY OF THE INVENTION

The present invention is directed to mutant RNA polymerases with particular deletions that confer resistance to various reagents, including phosphate, pyrophosphate, sodium chloride, and/or single stranded DNA.

Thus, in some embodiments, mutant polymerases are provided that comprise a deletion of at least four amino acids among the amino acids at positions corresponding to 167-174 of SEQ ID NO:1.

Also provided are mutant polymerases having greater resistance to 30 mM NaCl, 7.5 mM phosphate, or 20 μg/ml single stranded DNA than a wild-type T7 RNA polymerase having SEQ ID NO:1 or a wild-type T3 RNA polymerase having SEQ ID NO:3.

Additionally, nucleic acids comprising a nucleotide sequence encoding any of the above mutant polymerases are provided.

Further provided are vectors comprising the above nucleic acids.

In other embodiments, host cells transformed with the above vectors are provided.

In additional embodiments, methods of amplifying mRNA are provided. The methods comprise (a) combine the mRNA with a reverse transcriptase and an appropriate first buffer and first reagents to form a first mixture and incubate the first mixture under conditions and for a time sufficient to synthesize a first strand of a cDNA; (b) form a second mixture by adding (i) DNA polymerase or an RNA polymerase having DNA polymerase activity and (ii) an appropriate second buffer and second reagents to the first mixture comprising the first strand cDNA, and incubating the second mixture under conditions and for a time sufficient to synthesize a second strand of the cDNA and form a double stranded cDNA (ds-cDNA); and (c) form a third mixture by adding an appropriate third buffer, third reagents and any of the mutant polymerases described herein to the second mixture comprising the ds-cDNA, and incubating the third mixture under conditions and for a time sufficient to synthesize a needed amount of amplified RNA.

In further embodiments, a composition is provided. The composition comprises any of the mutant polymerases described herein, and a reagent at a concentration that is inhibitory to wild-type T7 RNA polymerase, wherein the reagent is a salt, phosphate, pyrophosphate or single stranded DNA.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** is a sequence alignment of the amino acid sequences of T3 RNA polymerase (SEQ ID NO:3) and T7 RNA polymerase (SEQ ID NO:1).

FIG. **2** is a graph comparing relative transcription activity of wild-type T7 polymerase with the mutant polymerase T7Δ8 in the presence of varying concentrations of potassium phosphate.

FIG. **3** is a graph comparing relative transcription activity of wild-type T7 polymerase with the mutant polymerase T7Δ8 in the presence of varying concentrations of NaCl.

DETAILED DESCRIPTION OF THE INVENTION

As used herein, the singular forms "a", "an" and "the" are intended to include the plural forms as well, unless the context clearly indicates otherwise. Additionally, the use of "or" is intended to include "and/or", unless the context clearly indicates otherwise.

The present invention is based in part on the discovery of a region of phage RNA polymerases that confer susceptibility to reagents that are commonly used in molecular biology protocols, such as phosphate, pyrophosphate, sodium chloride (NaCl), and single stranded DNA (ssDNA). That region is defined by amino acids 167-174 of T7 RNA polymerase ("T7"; SEQ ID NO:1) or corresponding regions of related phage RNA polymerases. As such, deletions of at least four amino acids in that region are useful in imparting resistance to those reagents. See Example 1, demonstrating that a mutant of T7 having a deletion of amino acids 167-174 of SEQ ID NO:1 is more resistant to phosphate, pyrophosphate, NaCl, and ssDNA than wild type T7. That mutant, designated T7Δ8, has the amino acid sequence of SEQ ID NO:2. It is believed that a deletion of any 4 of the amino acids in positions corresponding to 167-174 would result in an RNA polymerase that is more resistant to phosphate, pyrophosphate, NaCl, or single stranded DNA than wild type T7 RNA polymerase

Thus, in some embodiments, mutant polymerases are provided that comprise a deletion of at least four amino acids among the amino acids at positions corresponding to 167-174 of SEQ ID NO:1.

As used herein, amino acids corresponding to 167-174 of SEQ ID NO:1 are the amino acid residues from a second RNA polymerase sequence that align with amino acids 167-174 of SEQ ID NO:1 when SEQ ID NO:1 is aligned with the amino acid sequence of the second RNA polymerase using the computer program BLASTP 2.2.24+ or an equivalent program (Altschul et al., 1997; 2005). In such an alignment, it is recognized that there may not be 8 amino acid residues from the second RNA polymerase that align with 167-174 of SEQ ID NO:1, since the BLASTP program may confer a gap in the 167-174 region either in SEQ ID NO:1 (in which case there would be more than 8 corresponding amino acids) or in the sequence of the second RNA polymerase in that area (in which case there would be less than 8 corresponding amino acids).

In various embodiments, the mutant RNA polymerase comprises a deletion of 5, 6, 7 or 8 amino acids among the amino acids at positions corresponding to 167-174 of SEQ ID NO:1.

Numerous naturally occurring RNA polymerases have sufficient homology to SEQ ID NO:1 (e.g., at least 30%, at least 32%, at least 37%, at least 38%, at least 50%, at least 60%, at least 70%, or at least 80%) that the skilled artisan would understand that those polymerases have a region functionally equivalent to the region at 167-174 of SEQ ID NO:1. Table 1 provides a listing of such known RNA polymerases, including the homology of those RNA polymerases to T7 (SEQ ID NO:1) as well as the number of residues at the region equivalent to amino acids 167-174 of SEQ ID NO:1 that are identical, or identical + having conserved substitutions to that region of SEQ ID NO:1. As is known in the art, a "conserved substitution" is a substitution of an amino acid with another amino acid having a similar side chain. A conserved substitution would be a substitution with an amino acid that makes the smallest change possible in the charge of the amino acid or size of the side chain of the amino acid (alternatively, in the size, charge or kind of chemical group within the side chain) such that the overall peptide retains its spacial and charge conformation. For example, common conserved changes are Asp to Glu, Asn or Gln; His to Lys, Arg or Phe; Asn to Gln, Asp or Glu and Ser to Cys, Thr or Gly. For the purpose of the conserved substitution, the 20 essential amino acids can be grouped as follows: alanine, valine, leucine, isoleucine, proline, phenylalanine, tryptophan and methionine having nonpolar side chains; glycine, serine, threonine, cystine, tyrosine, asparagine and glutamine having uncharged polar side chains; aspartate and glutamate having acidic side chains; and lysine, arginine, and histidine having basic side chains.

TABLE 1

Comparison of T7 RNA polymerase with other RNA polymerases[1]				
Source	Genbank #	Homology to T7[2]	Identity to T7 at 167-174[3]	Identity + conserved substitutions at 167-174[4]
Enterobacteria 13a	ACF15888.1	98%	8/8	8/8
Yersinia pestis ΦA1122	AAP20500	98%	8/8	8/8
Salmonella Vi06	CBV65202.1	93%	8/8	8/8
Salmonella ΦSG-JL2	ACD75668.1	82%	7/8	7/8
Yersinia ΦY303-12	CAB63592.1	82%	7/8	7/8
Enterobacteria T3	CAC86264.1	82%	7/8	7/8
Enterobacteria 285P	ACV32460.1	76%	6/8	7/8
Kluyvera Kvp1	ACJ14548.1	76%	6/8	7/8
Enterobacteria BA14	ACF15731.1	76%	6/8	7/8
Yersinia Berlin	CAJ70654.1	75%	6/8	7/8
Yersinia Yep2	ACF15684.1	75%	6/8	7/8
Klebsiella K11	ACF15837.1	73%	5/8	7/8
Enterobacteria K11	CAA37330.1	72%	5/8	7/8
Morganella MmP1	ACY74627.1	71%	6/8	6/8
Enterobacteria K1F	AAZ72968.1	62%	4/8	5/8
*Enterobacteria*EcoDS1	ACF15785.1	62%	4/8	5/8
Vibrio N4	ACR16468.1	61%	3/8	5/8
Vibrio VP4	AAY46276.1	61%	3/8	5/8
Pseudomonas gh-1	AAO73140.1	57%	2/8	5/8
P. pudita KT2440	AAN67879.1	38%	4/8	5/8
Agrobacterium tumefaciens C58*	AAK86987.1	37%	3/8	4/8
Azorhizobium caulinodans ORS 571*	BAF89605.1	35%	0/8	3/8
Enterobacteria SP6	AAR90000.1	32%	1/8	3/8
Enterobacteria Sf6	CAA68288.1	32%	1/8	3/8
Burkholderia thailandensis MSMB43*	ZP_02468154.1	32%	1/8	3/8
Enterobacteria K1-5	AAL86891.1	32%	1/8	3/8
Enterobacteria K1E	CAJ29407.1	32%	1/8	3/8
Ralstonia RSB1	BAG70384.1	31%	2/8	4/8
Xanthomonas ΦL7	ACE75775.1	31%	1/8	3/8
Erwinia Era103	ABM63398.1	31%	1/8	3/8
Pyramidobacter piscolens W5455*	EFB89737.1	30%	1/8	1/8

[1]RNA polymerases having at least 30% amino acid identity to T7 when aligned using BLASTP 2.2.24+. All RNA polymerases listed are bacteriophage polymerases, unless indicated by an asterisk (*).
[2]Amino acid identity to T7 when aligned using BLASTP 2.2.24+.
[3]Number of identical amino acid residues to T7 at region corresponding to 167-174 of wild-type T7 when the amino acid sequence is aligned to the wild-type T7 amino acid sequence using BLASTP 2.2.24+.
[4]Number of identical and conserved amino acid residues to T7 at region corresponding to 167-174 of wild-type T7 when the amino acid sequence is aligned to the wild-type T7 amino acid sequence using BLASTP 2.2.24+.

A commonly used RNA polymerase that is closely related to T7 is T3 RNA polymerase ("T3"), having the amino acid sequence of SEQ ID NO:3. As indicated in Table 1, T3 is 82% identical to T7 and has 7 of 8 amino acids identical to 167-174 of T7 in the region corresponding thereto. See FIG. **1**, showing an alignment of the amino acid sequences of T3 with T7 (SEQ ID NO:3 and SEQ ID NO:1, respectively), where amino acids 167-174 (deleted in T7Δ8) of T7 and the corresponding region of T3 (amino acids 168-175) are bold-underlined. As such, the skilled artisan would understand that a deletion of 4, 5, 6, 7 or 8 amino acids of T3 within the region corresponding to 167-174 of T7 (i.e., within amino acids 168-175 of SEQ ID NO:3) would confer greater resistance to phosphate, pyrophosphate, NaCl, and/or ssDNA than wild type T3. Thus, in determining whether an RNA polymerase has a region corresponding to 167-174 of T7 to assess whether a deletion could be made to confer resistance to phosphate, pyrophosphate, NaCl, and/or ssDNA, the skilled artisan could evaluate the homology of the RNA polymerase in question with either T7 (SEQ ID NO:1) or T3 (SEQ ID NO:3). Thus, in some embodiments, the mutant polymerase of the present invention comprises an amino acid sequence having at least about 30% amino acid homology to SEQ ID NO:1 or SEQ ID NO:3. In other embodiments, the mutant polymerase comprises an amino acid sequence having at least about 50% amino acid homology to SEQ ID NO:1 or SEQ ID NO:3. In still other embodiments, the mutant polymerase comprises an amino acid sequence having at least about 60% amino acid homology to SEQ ID NO:1 or SEQ ID NO:3. In further embodiments, the mutant polymerase comprises an amino acid sequence having at least about 70% amino acid homology to SEQ ID NO:1 or SEQ ID NO:3. In still further embodiments, the mutant polymerase comprises an amino acid sequence having at least about 80% amino acid homology to SEQ ID NO:1 or SEQ ID NO:3. In even further embodiments, the mutant polymerase comprises an amino acid sequence having at least about 90% amino acid homology to SEQ ID NO:1 or SEQ ID NO:3. In additional embodiments, the mutant polymerase comprises an amino acid sequence having at least about 95% amino acid homology to SEQ ID NO:1 or SEQ ID NO:3. In still additional embodiments, the mutant polymerase comprises an amino acid sequence having at least about 98% amino acid homology to SEQ ID NO:1 or SEQ ID NO:3. In still further embodiments, the mutant polymerase comprises an amino acid sequence having at least about 99% amino acid homology to SEQ ID NO:1 or SEQ ID NO:3. Further, the mutant polymerase can comprise the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:3, except for the deletion.

When determining whether any particular RNA polymerase has a region functionally equivalent to the region at

167-174 of SEQ ID NO:1, the skilled artisan might also take into account the number of amino acids that are identical, or identical + conserved to 167-174 of SEQ ID NO:1 at the corresponding region of the RNA polymerase in question. For example, the skilled artisan would consider an RNA polymerase with at least ⅛, at least 2/8, at least ⅜, at least 4/8, at least ⅝, at least 6/8, or at least ⅞ identical, or identical + conserved amino acids, or any combination thereof, in the region corresponding to 167-174 of SEQ ID NO:1 as strong evidence that the corresponding region of that RNA polymerase is functionally equivalent to 167-174 of SEQ ID NO:1, such that a deletion of at least 4 amino acids in that corresponding region of the RNA polymerase in question would be likely to confer resistance to phosphate, pyrophosphate, NaCl and/or ssDNA.

In various embodiments, the mutant polymerase of the present invention is more resistant to phosphate, pyrophosphate, NaCl, and/or ssDNA than the same polymerase not having the deletion in the region corresponding to 167-174 of SEQ ID NO:1. In some of these embodiments, the polymerase has greater resistance to 30 mM, or 20 mM, or 10 mM or 5 mM NaCl; 10 mM, or 7.5 mM, or 5 mM, or 2.5 mM, or 1 mM phosphate; 10 mM, or 7.5 mM, or 5 mM, or 2.5 mM, or 1 mM pyrophosphate; or 20 μg/ml ssDNA than the same polymerase not having the deletion. In other of these embodiments, the polymerase has greater resistance to 30 mM NaCl, 7.5 mM phosphate, 7.5 mM pyrophosphate, or 20 μg/ml ssDNA than wild-type T7 RNA polymerase having SEQ ID NO:1 or wild-type T3 RNA polymerase having SEQ ID NO:3.

The mutants of the present invention also have the property that competition with promoter-independent synthesis by single-stranded DNA is reduced compared to the wild type enzyme. In some cases, not only is synthesis resistant to inhibition by single-stranded DNA competitor but there may be a stimulatory effect for synthesis of the legitimate promoter-driven target.

In some embodiments, the mutant polymerase further comprises at least one additional mutation. The additional mutation can be any mutation now known or later discovered. Nonlimiting examples of known useful mutations that can be present in the mutant polymerase of the present invention include mutations corresponding to the following mutations in SEQ ID NO:1: Y639F, S641A, F644Y, F667Y, E222K, S430P, F849I, F880Y, S633P, P266L, N748D, N748Q, Q758C and R756M. Some of these mutant polymerases have DNA polymerase activity, for example as conferred by mutations corresponding to Y639F and S641A of SEQ ID NO:1. Such mutant polymerases can be used for DNA sequencing by methods known in the art.

Also provided herein are mutant polymerases having greater resistance to 30 mM NaCl, 7.5 mM phosphate, 7.5 mM pyrophosphate, or 20 μg/ml single stranded DNA than a wild-type T7 RNA polymerase having SEQ ID NO:1 or a wild-type T3 RNA polymerase having SEQ ID NO:3. See Example 1. These mutant polymerases could have resistance to all of 30 mM NaCl, 7.5 mM phosphate, 7.5 mM pyrophosphate, and 20 μg/ml single stranded DNA (as T7Δ8 does) or to any one, two or three of these reagents. In some of these embodiments, the mutant polymerase has greater resistance to 30 mM NaCl than a wild-type T7 RNA polymerase having SEQ ID NO:1 or a wild-type T3 RNA polymerase having SEQ ID NO:3. In other embodiments, the mutant polymerase has greater resistance to 7.5 mM phosphate than a wild-type T7 RNA polymerase having SEQ ID NO:1 or a wild-type T3 RNA polymerase having SEQ ID NO:3. In additional embodiments, the mutant polymerase has greater resistance to pyrophosphate than a wild-type T7 RNA polymerase having SEQ ID NO:1 or a wild-type T3 RNA polymerase having SEQ ID NO:3. In still other embodiments, the mutant polymerase has greater resistance to 20 μg/ml ssDNA than a wild-type T7 RNA polymerase having SEQ ID NO:1 or a wild-type T3 RNA polymerase having SEQ ID NO:3.

The present invention is also directed to nucleic acids comprising a nucleotide sequence encoding any of the above-described mutant polymerases, i.e., a gene encoding the mutant polymerase. The nucleotide sequence can comprise any portion of a naturally occurring nucleotide sequence (e.g., the sequence of T7 or T3 RNA polymerase as encoded in the naturally occurring T7 or T3 bacteriophage, as provided in Genbank accessions M38308.1 and X02981.1, respectively, or the sequence of any of the enzymes in Table 1). Due to the redundancy in the various codons that code for specific amino acids, the nucleic acids that encode for the mutant polymerases can be comprised of substantially or even entirely a non-naturally occurring sequence. The nucleic acids comprising the nucleotide sequence can be DNA, RNA or analogs thereof. Examples of nucleic acid analogs include peptide nucleic acids, morpholino or locked nucleic acids, glycol nucleic acids or threose nucleic acids, as they are known in the art.

The above nucleic acids comprising a nucleotide sequence encoding the mutant polymerases can be combined with other nucleic acids, e.g., promoters, enhancers, antibiotic resistance genes etc. using well known methods of molecular biology. In some aspects, these combinations of nucleic acids form a vector that allows the above-described genes for the mutant polymerase to be transferred to a living cell, where the gene replicates in the cell. Thus, vectors are provided that comprise nucleic acids comprising a gene for any of the above-described mutant polymerases. The vectors can be any type known in the art, including but not limited to plasmid vectors, viral vectors, cloning vectors, shuttle vectors, or expression vectors.

Expression vectors are defined herein as DNA sequences that are required for the transcription of cloned copies of the mutant polymerase gene and the translation of its mRNA in an appropriate host.

Thus, DNA encoding the mutant polymerases may be subcloned into an expression vector for expression in a recombinant host cell. Recombinant host cells may be prokaryotic or eukaryotic, including but not limited to bacteria such as *E. coli*, plant cells, fungal cells such as yeast, mammalian cells including but not limited to cell lines of human, bovine, porcine, monkey and rodent origin, and insect cells including but not limited to *Drosophila* and silkworm derived cell lines as they are known in the art.

The expression vector may be introduced into host cells via any one of a number of techniques including but not limited to transformation, transfection, protoplast fusion, lipofection, and electroporation. The expression vector-containing cells are clonally propagated and individually analyzed to determine whether they produce the mutant polymerase protein.

Mutant polymerase-expressing host cell clones may be identified by any of several known means, including but not limited to immunological reactivity with anti-polymerase antibodies, or the presence of host cell-associated polymerase activity. Host cells transformed with any of the above-identified vectors are thus further provided herein. These host cells can be of any origin, including bacterial, archaeal, plant, fungal, insect or mammalian origin.

These mutant RNA polymerases can be used in any methods where such polymerases are used, including but not limited to synthesizing RNA probes, sequencing (particularly using the mutant polymerases that have DNA polymerase activity, as described above), and amplifying mRNA. The mutant polymerases that are resistant to phosphate, pyrophosphate, dsDNA, and/or NaCl are particularly useful for the latter methods, since inhibition of wild-type RNA polymerases by those reagents can be a concern in RNA amplification.

Thus, in additional embodiments, methods of amplifying mRNA are provided.

In additional embodiments, methods of amplifying mRNA are provided. The methods comprise (a) combine the mRNA with a reverse transcriptase and an appropriate first buffer and first reagents to form a first mixture and incubate the first mixture under conditions and for a time sufficient to synthesize a first strand of a cDNA; (b) form a second mixture by adding (i) DNA polymerase or an RNA polymerase having DNA polymerase activity and (ii) an appropriate second buffer and second reagents to the first mixture comprising the first strand cDNA, and incubating the second mixture under conditions and for a time sufficient to synthesize a second strand of the cDNA and form a double stranded cDNA (ds-cDNA); and (c) form a third mixture by adding an appropriate third buffer, third reagents and any of the mutant polymerases described herein to the second mixture comprising the ds-cDNA, and incubating the third mixture under conditions and for a time sufficient to synthesize a needed amount of amplified RNA.

In some of these embodiments, the first reagents comprise an oligo(dT)-T7 promoter, dATP, dCTP, dTTP, dGTP, and RNase inhibitor, the second reagents comprise RNase, and the third reagents comprise ATP, GTP, CTP, UTP and RNase inhibitor. In other of these embodiments, the second mixture is heat treated after the ds-cDNA is formed to denature the enzymes present therein. In some aspects, the mixture of step (c) further comprises pyrophosphatase. In other aspects, the mixture of step (c) further comprises a labeled nucleotide. In additional aspects, the mixture of step (c) further comprises pyrophosphatase and a labeled nucleotide.

The mutant polymerase of these embodiments can be any of the mutant polymerases described above, for example a mutant polymerase comprising the amino acid sequence of SEQ ID NO:2.

In various aspects of these embodiments, the DNA polymerase or RNA polymerase having DNA polymerase activity of step (b) is the mutant polymerase described above that has DNA polymerase activity (e.g., the mutant polymerase having mutations corresponding to Y639F and S641A of SEQ ID NO:2).

These methods are particularly useful where the cDNA is not purified before step (c) and where the method is performed in one container without removal of any mixture before the amplified RNA is synthesized, since the mutant polymerases described herein are resistant to reagents that are generally present in concentrations that are inhibitory to wild-type RNA polymerases.

In further embodiments, a composition is provided. The composition comprises any of the mutant polymerases described herein, and a reagent at a concentration that is inhibitory to wild-type T7 RNA polymerase. In these compositions, the reagent is a salt, phosphate, pyrophosphate or single stranded DNA. In some aspects, the reagent is a salt, for example NaCl, KCl, or any other salt that may be present in such a composition, where the salt is inhibitory to wild-type T7 RNA polymerase. The salt can be at any inhibitory concentration, for example 10 mM, 20 mM, 30 mM, any concentration between these concentrations, or any concentration above 30 mM. In other aspects, the reagent is phosphate or pyrophosphate, for example at 1 mM, 2 mM, 5 mM, 10 mM, any concentration between these concentrations, or any concentration above 10 mM. In further aspects the reagent is ssDNA, for example at 5 μg/ml, 10 μg/ml, 15 μg/ml, 20 μg/ml, any concentration between these concentrations, or any concentration above 20 μg/ml.

The mutant polymerase of these compositions can be any of the mutant polymerases described above, for example a mutant polymerase comprising the amino acid sequence of SEQ ID NO:2.

In some of these embodiments, the composition comprises a cDNA and reagents appropriate to transcribe the cDNA into RNA. For example the composition can be the mixture of step (c) in the methods of amplifying RNA described above.

In other of these embodiments, the composition is in a kit, where the mutant polymerase and reagent are in separate containers or the same container as appropriate. In some of these kits, the kit further comprises reagents, buffers and/or enzymes for amplifying mRNA by the method described above. For example, such kits, may comprise a reverse transcriptase and/or a DNA polymerase, and/or, e.g., any combination of an oligo(dT)-T7 promoter, dATP, dCTP, dTTP, dGTP, an RNase inhibitor, RNase, ATP, GTP, CTP, and/or UTP. Again, these reagents can be in separate containers or mixed together in any combination of containers. Instructions as appropriate may also be included in these kits.

Preferred embodiments are described in the following examples. Other embodiments within the scope of the claims herein will be apparent to one skilled in the art from consideration of the specification or practice of the invention as disclosed herein. It is intended that the specification, together with the examples, be considered exemplary only, with the scope and spirit of the invention being indicated by the claims, which follow the examples.

EXAMPLE 1

Isolation and Characterization of a T7 RNA Polymerase that is Resistant to Phosphate

The T7 RNA polymerase gene was fused to a histidine tag for ease of purification as described by Ellinger and Ehricht (1998), in the expression vector pQE30 (Qiagen). This plasmid, pQE30-T7, was used for all subsequent modifications.

Mutations were generated in the plasmid pQE30-T7 using PCR generated mutations. The following primers were used to amplify pQE30-T7 and generate mutations:

```
                                    (SEQ ID NO: 4)
F-T7d  pGTAGGGCACGTCTACAAGAAAG
       binds pQE30-T7 at bases 667-688.

                                    (SEQ ID NO: 5)
R-T7d  pGTTGAGTTGTTCCTCAACGTTTTTC
       binds pQE30-T7 at 636-660.
```

The mutagenic amplification was performed using the following mixture:

10 ng pQE30-T7

160 nM F-T7d

160 nM R-T7d

200 μM each of dATP, dCTP200, dGTP and TTP

2.5 units PfuUltra Hotstart DNA Polymerase

PfuUltra buffer, supplied by the manufacturer (Stratagene, La Jolla, Calif.).

This mixture was heated to 94° C. for two minutes then cycled 15 times using a cycle of 94° C. 20 seconds, 55.2° C.

20 seconds, 72° C. 6 minutes 10 seconds. This was followed by an extension at 72° C. for 5 minutes. After synthesis, starting template was removed by digestion with the restriction endonuclease DpnI (New England Biolabs, Ipswich, Mass.), which cleaves only the methylated starting DNA. The resulting DNA was separated by agarose gel (0.7%) followed by purification from the gel using the Qiagen QiaQuick gel extraction kit (Qiagen, Valencia, Calif.). The purified DNA was ligated using Quick Ligase (New England BioLabs, Ipswich, Mass.) as recommended by the manufacturer. The ligated DNA was used to transform the *Escherichia coli* strain Top10F' (Life Technologies, Carlsbad, Calif.). Several colonies grew on LB plates (Davis et al., 1980) containing 100 μg/ml ampicillin. Individual colonies were isolated, and the DNA was isolated from those colonies using standard techniques (Sambrook and Russell, 2001). Agarose gel electrophoresis was used to identify plasmids with large deletions that are unlikely to express active T7 RNA polymerase.

Strains containing the correct size plasmid were grown and induced in small cultures, and the modified T7 RNA Polymerases were purified using small Nickel-NTA columns (Qiagen, Valencia, Calif.) using standard techniques (Elinger and Ehricht, 1998). One mutation was chosen for further study.

Determining the Sequence of the Altered T7 RNA Polymerase.

The entire gene for the T7 RNA Polymerase that was chosen for further study was sequenced. Only a single mutation was found, a deletion of 24 base pairs, causing a deletion of 8 amino acid residues in the protein. References to peptide sequence of T7 RNA polymerase follow the numbering of amino acids residues as described by Dunn and Studier (1981) and Stahl and Zinn (1981) and as provided in SEQ ID NO:1. The amino acid residues missing in the enhanced T7 RNA polymerase described above are residues 167 to 174. This is adjacent to a region known to be involved in transcription termination. The sequence that is deleted in the modified T7 RNA polymerase is EEQLNKRV (SEQ ID NO:6).

Standard Transcription Assay.

The plasmid transcription template used in this work is pTAN, a plasmid containing a neomycin resistance gene after a T7 promoter that is linearized using the restriction enzyme PvuII. The run-off transcript that is produced from this plasmid is 790 bases long. The transcription reactions described herein contain

Template DNA (varying amounts)
80 mM Tris-HCl, pH 7.9
10 mM DTT
12 mM $MgCl_2$
1.5 mM Spermidine
10 mM NaCl
200 μg/ml BSA
3.75 mM each of UTP, ATP, CTP and GTP
2000 u/ml RNase Inhibitor (2000 units/ml)
12 u/ml Pyrophosphatase
T7 RNA Polymerase (amount varies).

If labeled nucleotides are used, the modified nucleotide replaces one fourth of the cognate nucleotide.

The reactions are assembled at room temperature then incubated at 37° C. for various times. Reactions are stopped by the addition of EDTA to 20 mM.

Quantifying a Single Transcription Product.

To determine the relative yield of a transcription reaction, the products of the transcription reactions were separated using a 1.2% Lonza flash gel (Lonza, Basel, Switzerland) as recommended. The gel was photographed using a Kodak 440 image scanner and a 523 nm cut-off filter. RNA specific bands were outlined using the manual region of interest (ROI) function in the Kodak Molecular Imaging software (version 4.04). The same size ROI was used for each band and a control area with no RNA was used as background. The NET intensity is calculated as the intensity of the RNA band with the background intensity subtracted. The intensity values were compared on a single gel, but not between gels.

Determining the Effect of Excess Single-Stranded DNA on Transcription Using the Modified T7 RNA Polymerase.

The inventors desired a novel RNA polymerase that would be resistant to excess single-stranded DNA, as commonly occurs in many molecular biology techniques (VanGelder et al., 1990). Table 2 shows the relative amount of specific product produced in a standard transcription reaction using 1.25 μg/ml plasmid template, and 100 units per ml T7 RNA polymerase in a 20 μl reaction at 37° C. for 18 hours. Single-stranded Salmon sperm DNA was used to test the sensitivity of T7 RNA polymerase to ssDNA.

TABLE 2

Relative transcription activity of wild-type T7 polymerase with the mutant polymerase T7Δ8 in the presence or absence of 20 μg/ml single stranded DNA

Polymerase	excess DNA	Relative Activity
wild-type T7	no	100%
wild-type T7	20 μg/ml	71%
T7 Δ8	no	100%
T7 Δ8	20 μg/ml	172%

With wild-type T7 RNA polymerase, the ssDNA inhibited synthesis of the template specific product. The mutant T7 RNA polymerase, T7Δ8, appeared to be stimulated in the presence of ssDNA.

Determining the Effect of Excess Phosphate or Pyrophosphate on Transcription Using the Modified T7 RNA Polymerase.

During transcription, as the RNA chain is elongated, pyrophosphate is produced. Pyrophosphate is broken down into phosphate by either endogenous enzymes, chemical reactions or by the addition of pyrophosphatase (Cunningham and Olfengand 1990). Phosphate can inhibit the transcription reaction, so the T7Δ8 mutant was tested for inhibition by phosphate. Table 3 shows the relative amount of specific product produced in a standard transcription reaction using 1.25 μg/ml plasmid template, and 100 units per ml enzyme in a 20 μl reaction at 37° C. for 18 hours. The phosphate or pyrophosphate was added in the form of a sodium salt at pH 8.0.

TABLE 3

Relative transcription activity of wild-type T7 polymerase and polymerase T7Δ8 in the presence or absence of 7.5 mM pyrophosphate or phosphate

Polymerase	no addition	7.5 mM pyrophosphate	7.5 mM phosphate
Wild-type T7	100%	71%	73%
T7Δ8	100%	139%	204%

With wild-type T7 RNA polymerase, the excess phosphate or pyrophosphate inhibited synthesis of the template specific product. The mutant T7 RNA polymerase, T7Δ8, appeared to be stimulated in the presence of excess phosphate. The stimulation by pyrophosphate may be due to contaminating phosphate in the pyrophosphate preparation due to breakdown of the pyrophosphate into phosphate.

FIG. 2 shows the relative amount of specific product produced in a standard transcription reaction using 0.625 μg/ml plasmid template, and 100 units per ml enzyme in a 20 μl reaction at 37° C. for 18 hours in the standard buffer with various concentrations of potassium phosphate added. The mutant T7 RNA polymerase was stimulated by phosphate up to about 5 mM, while the unmodified enzyme was inhibited by all phosphate concentrations tested.

Table 4 shows the relative amount of specific product produced in a standard transcription reaction using 2 μg/ml plasmid template and 500 units per ml enzyme at 37° C. for 18 hours in the standard buffer with various concentrations of potassium phosphate or pyrophosphate added.

TABLE 4

Relative transcription activity of wild type T7 polymerase and polymerase T7Δ8 in the presence or absence of varying concentrations of pyrophosphate or phosphate

	Wild-Type	T7Δ8
0 mM Pi	1.00	1.00
10 mM Pi	0.47	0.69
5 mM Pi	0.69	1.03
20 mM PPi	0.16	0.16
10 mM PPi	0.52	0.52
5 mM PPi	0.78	1.16

Determining the Effect of High Salt (NaCl) on Transcription Using the Modified T7 RNA Polymerase.

High salt concentrations are known to inhibit transcription from many bacteriophage RNA polymerases, such as T7, SP6 and T3 (Milburn et al., 1993, Milburn et al. 2003). T7Δ8 was compared with wild-type T7. FIG. **3** shows the relative amount of specific product produced in a standard transcription reaction using 1.25 μg/ml plasmid template, and 200 units per ml enzyme in a 20 μl reaction at 37° C. for 18 hours in the standard buffer without added NaCl or with increasing concentrations of NaCl.

As shown in FIG. **3**, with wild-type T7 RNA polymerase, the excess NaCl inhibited synthesis of the template specific product. The mutant T7 RNA polymerase, T7Δ8, is not inhibited in the presence of 10 mM NaCl, and is generally less affected by salt.

EXAMPLE 2

Use of T7Δ8 for One Tube RNA Amplification Reactions

The mutant T7 polymerase described in Example 1 (T7Δ8) was compared with wild-type T7 (SEQ ID NO:1) in RNA amplification reactions performed in a single tube as follows.

First Strand Synthesis—5 μl

100 ng human reference RNA (Stratagene, La Jolla, Calif.)

2.5 μM T7T24 primer

2 mM each of dTTP, dCTP, dATP and dGTP

First Strand buffer: 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, and 5 mM DTT

100 units reverse transcriptase

100 units RNase Inhibitor

The above mixture was incubated at 42° C. for 2 h.

Second Strand Synthesis

After the first strand synthesis incubation, the following was added to the mixture to 25 potassium phosphate buffer, pH 7.0 to 20 mM

DNA Polymerase I (9 units)

RNase H (20 units)

This mixture was incubated at 16° C. for 2 h

After the second strand synthesis incubation, the enzymes were denatured by incubation at 65° C. for 10 min.

Transcription

Following the above steps, the following was added to the mixture to 75 μl:

7.5 μl 10× transcription buffer consisting of 800 mM Tris-HCl pH 7.9, 120 mM $MgCl_2$, 15 mM Spermidine, 100 mM NaCl, and 2 mg/ml BSA

7.5 μl 100 mM DTT

7.5 μl of a mixture of 37.5 mM ATP, 37.5 mM GTP, 25 mM CTP, 25 mM UTP, 12.5 mM Bio-11-CTP and 12.5 mM Bio-16-UTP

150 units Porcine RNase Inhibitor

0.9 units pyrophosphatase

250 units of wild type T7 RNA polymerase or 1250 units of T7Δ8

The reaction proceeded at 37° for 16 hours.

After transcription, the RNA produced was purified using Qiagen RNeasy mini columns.

The results of these studies are provided in Table 5. The standard deviations (SD) are based on two replications. As shown in Table 5, T7Δ8 provided an aRNA yield that compared favorably to wild-type T7.

TABLE 5

Yield of amplified RNA ("aRNA") prepared using wild-type T7 polymerase (WT) and polymerase T7Δ8 (Mut) in a one tube amplification method

Sample	μg aRNA	SD
Set 1 WT	21.2	0.5
Set 1 Mut	43.5	1.2
Set 2 WT	15.7	0.9
Set 2 Mut	31.3	0.3

Wild-type T7 and T7Δ8 were also compared for their ability to amplify RNA for use on arrays. The RNA amplification materials and procedures were as follows.

First Strand Synthesis—5 μl

100 ng human reference RNA, human colon RNA, or human thymus RNA

2.5 μM T7T24 primer

1 mM each of dTTP, dCTP, dATP and dGTP

First Strand buffer: 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, and 5 mM DTT

100 units reverse transcriptase

100 units RNase inhibitor

The above mixture was incubated at 42° C. for 2 h.

Second Strand Synthesis

After the first strand synthesis incubation, the following was added to the mixture to 25 μl: potassium phosphate buffer, pH 7.0 to 40 mM

$MgCl_2$ to 1 mM

DNA polymerase (9 units)

RNase H (20 units)

This mixture was incubated at 16° C. for 2 h

After the second strand synthesis incubation, the enzymes were denatured by incubation at 65° C. for 10 min.

Transcription

Following the above steps, the following was added to the mixture to 75 μl:

7.5 μl 10× transcription buffer consisting of 800 mM Tris-HCl pH 7.9, 120 mM $MgCl_2$, 15 mM spermidine, 100 mM NaCl, and 2 mg/ml BSA

7.5 μl 100 mM DTT
7.5 μl of a mixture of 37.5 mM ATP, 37.5 mM GTP, 25 mM CTP, 25 mM UTP, 12.5 mM Bio-11-CTP and 12.5 mM Bio-16-UTP
150 units porcine RNase inhibitor
0.9 units pyrophosphatase
250 units wild type T7 RNA polymerase or 1250 units of T7Δ8
The reaction proceeded at 37° for 16 hours.
After transcription, the RNA produced was purified using Qiagen RNeasy mini columns. The purified RNAs were applied to Affymetrix HG-U133a chips according to the manufacturer's protocols.

The results for the human reference RNA is provided in Table 6; the thymus and colon results are provided in Table 7.

TABLE 6

Comparison of expression data from human reference RNA amplified by wild-type T7 and T7Δ8

Human Reference RNA	WT	T7Δ8
% P	51.6	51.5
% M	1.9	1.9
GAPDH 3'/5'	0.98	1.01
GAPDH 3'/M	1.06	1.13
Actin 3'/5'	1.93	1.94
Actin 3'/M	1.12	0.82
Scale Factor	0.97	1.08
$\boxed{R^2 = 0.977}$		

TABLE 7

Comparison of expression data from (a) human thymus RNA and (b) human colon RNA amplified by wild-type T7 and T7Δ8*

(a)			(b)		
Chip ID	WT T7 thymus 10 μg ECT5	T7Δ8 thymus 10 μg EMT6	Chip ID	WT T7 colon 7 μg ECC3	T7Δ8 colon 7 μg EMC4
% P	44.3	42.3	% P	42.7	44.3
% A	53.8	56	% A	55.3	53.8
% M	1.9	1.7	% M	1.9	1.9
Scale factor	2.283	2.62	Scale factor	3.34	3.47
Actin 3'/5'	2.22	3.08	Actin 3'/5'	9.69	5.96
Actin 3'/M	1.34	1.11	Actin 3'/M	2.6	1.51
GAPDH 3'/5'	1.17	1.22	GAPDH 3'/5'	2.92	2.61
GAPDH 3'/M	1.27	1.15	GAPDH 3'/M	2.28	2
$RSQ_{staining}$	0.996	0.994	$RSQ_{staining}$	0.987	0.992
	$R^2 = 0.983$			$R^2 = 0.979$	

*% P = percentage of probe sets deemed present in the RNA; % A = percentage of probe sets deemed absent in the RNA; % M = percentage of probe sets deemed marginally present; Scale factor = number that the signal is multiplied by to make the average value the same for all chips; RSQstaining is a measure of the evenness of the staining on the chip; the Actin and GAPDH measurements are relative amounts of these housekeeping genes present, using different probes as indicated; The R^2 values compare mutant to wild-type on the same RNA. Further information can be obtained in "GeneChip ® Expression Analysis" available at www.affymetrix.com.

As shown in Tables 6 and 7, the results using RNA amplified by wild-type T7 was very similar to the results using RNA amplified by T7Δ8, establishing that T7Δ8 can be substituted for wild-type T7 polymerase in procedures to produce high quality amplified RNA.

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European Patent Application No. EP1403364A1
U.S. Pat. No. 5,256,555.
U.S. Pat. No. 5,385,834.
U.S. Pat. No. 6,586,218.
U.S. Pat. No. 6,586,219.
U.S. Pat. No. 7,335,471.
U.S. Pat. No. 7,507,567.

In view of the above, it will be seen that the several advantages of the invention are achieved and other advantages attained.

As various changes could be made in the above methods and compositions without departing from the scope of the invention, it is intended that all matter contained in the above description and shown in the accompanying drawings shall be interpreted as illustrative and not in a limiting sense.

All references cited in this specification are hereby incorporated by reference. The discussion of the references herein is intended merely to summarize the assertions made by the authors and no admission is made that any reference constitutes prior art. Applicants reserve the right to challenge the accuracy and pertinence of the cited references.

APPENDIX

SEQ ID NOs

SEQ ID NO: 1 -
Wild type T7 RNA Polymerase - Genbank Accession NP041960.1
Residues deleted in SEQ ID NO: 2 - 167-174 - in bold underline

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  1  mntiniaknd fsdielaaip fntladhyge rlareqlale hesyemgear frkmferqlk
 61  agevadnaaa kplittllpk miarindwfe evkakrgkrp tafqflqeik peavayitik
121  ttlacltsad nttvqavasa igraiedear fgrirdleak hfkknveeql nkrvghvykk
181  afmqvveadm lskgllggea wsswhkedsi hvgvrcieml iestgmvslh rqnagvvgqd
241  setielapey aeaiatraga lagispmfqp cvvppkpwtg itgggywang rrplalvrth
301  skkalmryed vympevykai niaqntawki nkkvlavanv itkwkhcpve dipaiereel
361  pmkpedidmn pealtawkra aaavyrkdka rksrrislef mleqankfan hkaiwfpynm
421  dwrgrvyavs mfnpqgndmt kglltlakgk pigkegyywl kihgancagv dkvpfperik
481  fieenhenim acaksplent wwaeqdspfc flafcfeyag vqhhglsync slplafdgsc
541  sgiqhfsaml rdevggravn llpsetvqdi ygivakkvne ilqadaingt dnevvtvtde
601  ntgeisekvk lgtkalagqw laygvtrsvt krsvmtlayg skefgfrqqv ledtiqpaid
661  sgkglmftqp nqaagymakl iwesvsvtvv aaveamnwlk saakllaaev kdkktgeilr
721  krcavhwvtp dgfpvwqeyk kpiqtrinlm flgqfrlqpt intnkdseid ahkqesgiap
781  nfvhsqdgsh lrktvvwahe kygiesfali hdsfgtipad aanlfkavre tmvdtyescd
841  vladfydqfa dqlhesqldk mpalpakgnl nlrdilesdf afa
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SEQ ID NO: 2 -
Mutant T7Δ8

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  1  mntiniaknd fsdielaaip fntladhyge rlareqlale hesyemgear frkmferqlk
 61  agevadnaaa kplittllpk miarindwfe evkakrgkrp tafqflqeik peavayitik
121  ttlacltsad nttvqavasa igraiedear fgrirdleak hfkknvghvykk
173  afmqvveadm lskgllggea wsswhkedsi hvgvrcieml iestgmvslh rqnagvvgqd
233  setielapey aeaiatraga lagispmfqp cvvppkpwtg itgggywang rrplalvrth
293  skkalmryed vympevykai niaqntawki nkkvlavanv itkwkhcpve dipaiereel
353  pmkpedidmn pealtawkra aaavyrkdka rksrrislef mleqankfan hkaiwfpynm
413  dwrgrvyavs mfnpqgndmt kglltlakgk pigkegyywl kihgancagv dkvpfperik
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APPENDIX-continued

SEQ ID NOs
463 fieenhenim acaksplent wwaeqdspfc flafcfeyag vqhhglsync slplafdgsc
533 sgiqhfsaml rdevggravn llpsetvqdi ygivakkvne ilqadaingt dnevvtvtde
593 ntgeisekvk lgtkalagqw laygvtrsvt krsvmtlayg skefgfrqqv ledtiqpaid
653 sgkglmftqp nqaagymakl iwesysvtvv aaveamnwlk saakllaaev kdkktgeilr
713 krcavhwvtp dgfpvwqeyk kpiqtrlnlm flgqfrlqpt intnkdseid ahkqesgiap
773 nfvhsqdgsh lrktvvwahe kygiesfali hdsfgtipad aanlfkavre tmvdtyescd
833 vladfydqfa dqlhesqldk mpalpakgnl nlrdilesdf afa
SEQ ID NO: 3 -
Wild type T3 RNA Polymerase - Genbank Accession NP523301.1 Residues 168-175, corresponding to deleted residues in SEQ ID NO: 2, in bold underline
1 mniieniekn dfseielaai pfntladhyg salakeqlal ehesyelger rflkmlerqa
61 kageiadnaa akpllatllp klttrivewl eeyaskkgrk psayaplqll kpeasafitl
121 kvilasltst nmttiqaaag mlgkaiedea rfgrirdlea khfkkhv**<u>eeq lnkrh</u>**gqvyk
181 kafmqvvead migrgllgge awsswdkett mhvgirliem liestglvel qrhnagnags
241 dhealqlaqe yvdvlakrag alagispmfq pcvvppkpwv aitgggywan grrplalvrt
301 hskkglmrye dvympevyka vnlaqntawk inkkvlavvn eivnwkncpv adipslerqe
361 lppkpddidt neaalkewkk aaagiyrldk arvsrrisle fmleqankfa skkaiwfpyn
421 mdwrgrvyav pmfnpqgndm tkglltlakg kpigeegfyw lkihgancag vdkvpfperi
481 afiekhvddi lacakdpinn twwaeqdspf cflafcfeya gvthhglsyn cslplafdgs
541 csgiqhfsam lrdevggrav nllpsetvqd iygivaqkvn eilkqdaing tpnemitvtd
601 kdtgeisekl klgtstlaqq wlaygvtrsv tkrsvmtlay gskefgfrqq vlddtiqpai
661 dsgkglmftq pnqaagymak liwdaysvtv vaaveamnwl ksaakllaae vkdkktkeil
721 rhrcavhwtt pdgfpvwqey rkplqkrldm iflgqfrlqp tintlkdsgi dahkqesgia
781 pnfvhsqdgs hlrmtvvyah ekygiesfal ihdsfgtipa dagklfkavr etmvityenn
841 dvladfysqf adqlhetqld kmpplpkkgn lnlqdilksd fafa
SEQ ID NO: 4 -
Forward primer F-T7 GTAGGGCACGTCTACAAGAAAG
SEQ ID NO: 5 -
Reverse primer R-T7 GTTGAGTTGTTCCTCAACGTTTTTC
SEQ ID NO: 6 -
Amino acid residues deleted in T7Δ8. EEQLNKRV

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    50                  55                  60

Ala Asp Asn Ala Ala Ala Lys Pro Leu Ile Thr Thr Leu Leu Pro Lys
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Met Ile Ala Arg Ile Asn Asp Trp Phe Glu Glu Val Lys Ala Lys Arg
                85                  90                  95

Gly Lys Arg Pro Thr Ala Phe Gln Phe Leu Gln Glu Ile Lys Pro Glu
            100                 105                 110

Ala Val Ala Tyr Ile Thr Ile Lys Thr Thr Leu Ala Cys Leu Thr Ser
        115                 120                 125

Ala Asp Asn Thr Thr Val Gln Ala Val Ala Ser Ala Ile Gly Arg Ala
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Ile Glu Asp Glu Ala Arg Phe Gly Arg Ile Arg Asp Leu Glu Ala Lys
145                 150                 155                 160

His Phe Lys Lys Asn Val Glu Glu Gln Leu Asn Lys Arg Val Gly His
                165                 170                 175

Val Tyr Lys Lys Ala Phe Met Gln Val Val Glu Ala Asp Met Leu Ser
            180                 185                 190

Lys Gly Leu Leu Gly Gly Glu Ala Trp Ser Ser Trp His Lys Glu Asp
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Ser Ile His Val Gly Val Arg Cys Ile Glu Met Leu Ile Glu Ser Thr
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Arg Ala Gly Ala Leu Ala Gly Ile Ser Pro Met Phe Gln Pro Cys Val
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Asn Gly Arg Arg Pro Leu Ala Leu Val Arg Thr His Ser Lys Lys Ala
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Leu Met Arg Tyr Glu Asp Val Tyr Met Pro Glu Val Tyr Lys Ala Ile
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Asn Ile Ala Gln Asn Thr Ala Trp Lys Ile Asn Lys Lys Val Leu Ala
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Val Ala Asn Val Ile Thr Lys Trp Lys His Cys Pro Val Glu Asp Ile
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Pro Ala Ile Glu Arg Glu Glu Leu Pro Met Lys Pro Glu Asp Ile Asp
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Met Asn Pro Glu Ala Leu Thr Ala Trp Lys Arg Ala Ala Ala Ala Val
    370                 375                 380

Tyr Arg Lys Asp Lys Ala Arg Lys Ser Arg Arg Ile Ser Leu Glu Phe
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Met Leu Glu Gln Ala Asn Lys Phe Ala Asn His Lys Ala Ile Trp Phe
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Phe Ile Glu Glu Asn His Glu Asn Ile Met Ala Cys Ala Lys Ser Pro
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Gln Glu Tyr Lys Lys Pro Ile Gln Thr Arg Leu Asn Leu Met Phe Leu
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Gly Gln Phe Arg Leu Gln Pro Thr Ile Asn Thr Asn Lys Asp Ser Glu
        755                 760                 765

Ile Asp Ala His Lys Gln Glu Ser Gly Ile Ala Pro Asn Phe Val His
    770                 775                 780

Ser Gln Asp Gly Ser His Leu Arg Lys Thr Val Val Trp Ala His Glu
785                 790                 795                 800

Lys Tyr Gly Ile Glu Ser Phe Ala Leu Ile His Asp Ser Phe Gly Thr
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Ile Pro Ala Asp Ala Ala Asn Leu Phe Lys Ala Val Arg Glu Thr Met
            820                 825                 830

Val Asp Thr Tyr Glu Ser Cys Asp Val Leu Ala Asp Phe Tyr Asp Gln
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835 840 845

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Lys His Cys Pro Val Glu Asp Ile Pro Ala Ile Glu Arg Glu Glu Leu
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Pro Met Lys Pro Glu Asp Ile Asp Met Asn Pro Glu Ala Leu Thr Ala
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Trp Lys Arg Ala Ala Ala Ala Val Tyr Arg Lys Asp Lys Ala Arg Lys
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Ser Arg Arg Ile Ser Leu Glu Phe Met Leu Glu Gln Ala Asn Lys Phe
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Lys Gly Leu Leu Thr Leu Ala Lys Gly Lys Pro Ile Gly Lys Glu Gly
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Tyr Tyr Trp Leu Lys Ile His Gly Ala Asn Cys Ala Gly Val Asp Lys
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Val Pro Phe Pro Glu Arg Ile Lys Phe Ile Glu Glu Asn His Glu Asn
465                 470                 475                 480

Ile Met Ala Cys Ala Lys Ser Pro Leu Glu Asn Thr Trp Trp Ala Glu
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Gln Asp Ser Pro Phe Cys Phe Leu Ala Phe Cys Phe Glu Tyr Ala Gly
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Asp Ile Tyr Gly Ile Val Ala Lys Lys Val Asn Glu Ile Leu Gln Ala
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        675                 680                 685

Asn Trp Leu Lys Ser Ala Ala Lys Leu Leu Ala Ala Glu Val Lys Asp
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Lys Lys Thr Gly Glu Ile Leu Arg Lys Arg Cys Ala Val His Trp Val
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Thr Pro Asp Gly Phe Pro Val Trp Gln Glu Tyr Lys Lys Pro Ile Gln
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Thr Arg Leu Asn Leu Met Phe Leu Gly Gln Phe Arg Leu Gln Pro Thr
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Ile Asn Thr Asn Lys Asp Ser Glu Ile Asp Ala His Lys Gln Glu Ser
        755                 760                 765

Gly Ile Ala Pro Asn Phe Val His Ser Gln Asp Gly Ser His Leu Arg
    770                 775                 780

Lys Thr Val Val Trp Ala His Glu Lys Tyr Gly Ile Glu Ser Phe Ala
785                 790                 795                 800

Leu Ile His Asp Ser Phe Gly Thr Ile Pro Ala Asp Ala Ala Asn Leu
                805                 810                 815

Phe Lys Ala Val Arg Glu Thr Met Val Asp Thr Tyr Glu Ser Cys Asp
            820                 825                 830

Val Leu Ala Asp Phe Tyr Asp Gln Phe Ala Asp Gln Leu His Glu Ser
        835                 840                 845

Gln Leu Asp Lys Met Pro Ala Leu Pro Ala Lys Gly Asn Leu Asn Leu
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Glu Arg Arg Phe Leu Lys Met Leu Glu Arg Gln Ala Lys Ala Gly Glu
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Ile Ala Asp Asn Ala Ala Ala Lys Pro Leu Leu Ala Thr Leu Leu Pro
65                  70                  75                  80

Lys Leu Thr Thr Arg Ile Val Glu Trp Leu Glu Glu Tyr Ala Ser Lys
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Lys Gly Arg Lys Pro Ser Ala Tyr Ala Pro Leu Gln Leu Leu Lys Pro
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Glu Ala Ser Ala Phe Ile Thr Leu Lys Val Ile Leu Ala Ser Leu Thr
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Ser Thr Asn Met Thr Thr Ile Gln Ala Ala Ala Gly Met Leu Gly Lys
    130                 135                 140

Ala Ile Glu Asp Glu Ala Arg Phe Gly Arg Ile Arg Asp Leu Glu Ala
145                 150                 155                 160

Lys His Phe Lys Lys His Val Glu Glu Gln Leu Asn Lys Arg His Gly
                165                 170                 175

Gln Val Tyr Lys Lys Ala Phe Met Gln Val Val Glu Ala Asp Met Ile
            180                 185                 190

Gly Arg Gly Leu Leu Gly Gly Glu Ala Trp Ser Ser Trp Asp Lys Glu
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Thr Thr Met His Val Gly Ile Arg Leu Ile Glu Met Leu Ile Glu Ser
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                245                 250                 255

Lys Arg Ala Gly Ala Leu Ala Gly Ile Ser Pro Met Phe Gln Pro Cys
            260                 265                 270

Val Val Pro Pro Lys Pro Trp Val Ala Ile Thr Gly Gly Gly Tyr Trp
        275                 280                 285

Ala Asn Gly Arg Arg Pro Leu Ala Leu Val Arg Thr His Ser Lys Lys
    290                 295                 300

Gly Leu Met Arg Tyr Glu Asp Val Tyr Met Pro Glu Val Tyr Lys Ala
305                 310                 315                 320

Val Asn Leu Ala Gln Asn Thr Ala Trp Lys Ile Asn Lys Lys Val Leu
                325                 330                 335

Ala Val Val Asn Glu Ile Val Asn Trp Lys Asn Cys Pro Val Ala Asp
            340                 345                 350

Ile Pro Ser Leu Glu Arg Gln Glu Leu Pro Pro Lys Pro Asp Asp Ile
        355                 360                 365

Asp Thr Asn Glu Ala Ala Leu Lys Glu Trp Lys Lys Ala Ala Ala Gly
    370                 375                 380

Ile Tyr Arg Leu Asp Lys Ala Arg Val Ser Arg Arg Ile Ser Leu Glu
385                 390                 395                 400

Phe Met Leu Glu Gln Ala Asn Lys Phe Ala Ser Lys Lys Ala Ile Trp
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Phe Pro Tyr Asn Met Asp Trp Arg Gly Arg Val Tyr Ala Val Pro Met
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Phe Asn Pro Gln Gly Asn Asp Met Thr Lys Gly Leu Leu Thr Leu Ala
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Lys Gly Lys Pro Ile Gly Glu Glu Gly Phe Tyr Trp Leu Lys Ile His
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Gly Ala Asn Cys Ala Gly Val Asp Lys Val Pro Phe Pro Glu Arg Ile
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Ala Phe Ile Glu Lys His Val Asp Asp Ile Leu Ala Cys Ala Lys Asp
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Pro Ile Asn Asn Thr Trp Trp Ala Glu Gln Asp Ser Pro Phe Cys Phe
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Gln Lys Val Asn Glu Ile Leu Lys Gln Asp Ala Ile Asn Gly Thr Pro
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Asn Glu Met Ile Thr Val Thr Asp Lys Asp Thr Gly Glu Ile Ser Glu
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Lys Leu Lys Leu Gly Thr Ser Thr Leu Ala Gln Gln Trp Leu Ala Tyr
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Gly Val Thr Arg Ser Val Thr Lys Arg Ser Val Met Thr Leu Ala Tyr
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Gly Ser Lys Glu Phe Gly Phe Arg Gln Gln Val Leu Asp Asp Thr Ile
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Gln Pro Ala Ile Asp Ser Gly Lys Gly Leu Met Phe Thr Gln Pro Asn
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Gln Ala Ala Gly Tyr Met Ala Lys Leu Ile Trp Asp Ala Val Ser Val
        675                 680                 685

Thr Val Val Ala Ala Val Glu Ala Met Asn Trp Leu Lys Ser Ala Ala
    690                 695                 700

Lys Leu Leu Ala Ala Glu Val Lys Asp Lys Lys Thr Lys Glu Ile Leu
705                 710                 715                 720

Arg His Arg Cys Ala Val His Trp Thr Thr Pro Asp Gly Phe Pro Val
                725                 730                 735

Trp Gln Glu Tyr Arg Lys Pro Leu Gln Lys Arg Leu Asp Met Ile Phe
            740                 745                 750

Leu Gly Gln Phe Arg Leu Gln Pro Thr Ile Asn Thr Leu Lys Asp Ser
        755                 760                 765

Gly Ile Asp Ala His Lys Gln Glu Ser Gly Ile Ala Pro Asn Phe Val
    770                 775                 780

His Ser Gln Asp Gly Ser His Leu Arg Met Thr Val Val Tyr Ala His
785                 790                 795                 800

Glu Lys Tyr Gly Ile Glu Ser Phe Ala Leu Ile His Asp Ser Phe Gly
                805                 810                 815

Thr Ile Pro Ala Asp Ala Gly Lys Leu Phe Lys Ala Val Arg Glu Thr
            820                 825                 830

Met Val Ile Thr Tyr Glu Asn Asn Asp Val Leu Ala Asp Phe Tyr Ser
        835                 840                 845

Gln Phe Ala Asp Gln Leu His Glu Thr Gln Leu Asp Lys Met Pro Pro
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Leu Pro Lys Lys Gly Asn Leu Asn Leu Gln Asp Ile Leu Lys Ser Asp
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Phe Ala Phe Ala


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-continued

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What is claimed is:

1. A synthetically generated polymerase selected from (i) a protein having an amino acid sequence that is at least 90% homologous to the amino acid sequence of the polymerase of SEQ ID NO: 1 and a deletion of the 8 amino acids corresponding to residues 167-174 of SEQ ID NO: 1 and (ii) a protein having an amino acid sequence that is at least 90% homologous to the amino acid sequence of the polymerase of SEQ ID NO:3 and a deletion of the 8 amino acids corresponding to residues 168-175 of SEQ ID NO: 3.

2. The polymerase of claim **1**, having an amino acid sequence that is at least 95% homologous to the polymerase of SEQ ID NO: 1 or the polymerase of SEQ ID NO:3.

3. The polymerase of claim **1**, having an amino acid sequence that is at least 98% homologous to SEQ ID NO:1 or SEQ ID NO:3.

4. The polymerase of claim **1**, having an amino acid sequence that is at least 99% homologous to SEQ ID NO:1 or SEQ ID NO:3.

5. A polymerase having the amino acid sequence of SEQ ID NO: 2.

6. The polymerase of claim **1**, having greater resistance to 30 mM NaCl, 7.5 mM phosphate, 7.5 mM pyrophosphate, or 20 μg/ml single stranded DNA than the wild-type T7 RNA polymerase having the sequence of SEQ ID NO:1 or the wild-type T3 RNA polymerase having the sequence of SEQ ID NO:3.

7. The polymerase of claim **1**, further comprising at least one mutation corresponding to mutations in SEQ ID NO:1 selected from the group consisting of Y639F, S641A, F644Y, F667Y, E222K, S430P, F849I, F880Y, S633P, P266L, N748D, N748Q, Q758C and R756M.

8. The polymerase of claim **7**, comprising mutations corresponding to Y639F and S641A in SEQ ID NO:1.

9. The polymerase of claim **1** having greater resistance to 30 mM NaCl, 7.5 mM phosphate, or 20 μg/ml single stranded DNA than a wild-type T7 RNA polymerase having the sequence of SEQ ID NO:1 or a wild-type T3 RNA polymerase having the sequence of SEQ ID NO:3.

10. A method of amplifying mRNA comprising

(a) combining the mRNA with a reverse transcriptase and an appropriate first buffer and first reagents to form a first mixture and incubate the first mixture under conditions and for a time sufficient to synthesize a first strand of a cDNA;

(b) forming a second mixture by adding (i) DNA polymerase or an RNA polymerase having DNA polymerase activity and (ii) an appropriate second buffer and second reagents to the first mixture comprising the first strand cDNA, and incubating the second mixture under conditions and for a time sufficient to synthesize a second strand of the cDNA and form a double stranded cDNA (ds-cDNA); and

(c) forming a third mixture by adding an appropriate third buffer, third reagents and the polymerase of any one of claims **1**, **2**, **3**, **4**, **5**, **6**, **7**, **8** and **9** to the second mixture comprising the ds-cDNA, and incubating the third mixture under conditions and for a time sufficient to synthesize a needed amount of amplified RNA.

11. A composition comprising the polymerase of any one of claims **1**, **2**, **3**, **4**, **5**, **6**, **7**, **8** and **9**, and a reagent selected from a salt, phosphate, pyrophosphate or single stranded DNA at a concentration that is inhibitory to wild-type T7 RNA polymerase.

12. The composition of claim **11**, wherein said composition is in a kit, and the kit further comprises one or more reagents, buffers and/or enzymes for amplifying mRNA.

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